Effect of renin inhibition and AT1R blockade on myocardial remodeling in the transgenic Ren2 rat

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Effect of renin inhibition and AT1R blockade on myocardial remodeling in the transgenic Ren2 rat. Am J Physiol Endocrinol Metab 295: E103–E109, 2008. First published May 6, 2008; doi:10.1152/ajpendo.00752.2007.—Angiotensin II (Ang II) stimulation of the Ang type 1 receptor (AT1R) facilitates myocardial remodeling through NADPH oxidase-mediated generation of oxidative stress. Components of the renin-angiotensin system constitute an autocrine/paracrine unit in the myocardium, including renin, which is the rate-limiting step in the generation of Ang II. This investigation sought to determine whether cardiac oxidative stress and cellular remodeling could be attenuated by in vivo renin inhibition and/or AT1R blockade in a rodent model of chronically elevated tissue Ang II levels, the transgenic (mRen2)27 rat (Ren2). The Ren2 overexpresses the mouse renin transgene with resultant hypertension, insulin resistance, and cardiovascular damage. Young (6- to 7-wk-old) heterozygous (+/−) male Ren2 and age-matched Sprague-Dawley rats were treated with the renin inhibitor aliskiren, which has high preferential affinity for human and mouse renin, an AT1R blocker, irbesartan, or placebo for 3 wk. Myocardial NADPH oxidase activity and immunostaining for NADPH oxidase subunits and 3-nitrotyrosine were evaluated and remodeling changes assessed by light and transmission electron microscopy. Blood pressure, myocardial NADPH oxidase activity and subunit immunostaining, 3-nitrotyrosine, perivascular fibrosis, mitochondrial content, and markers of activity were significantly increased in Ren2 compared with SD littermates. Both renin inhibition and blockade of the AT1R significantly attenuated cardiac functional and structural alterations, although irbesartan treatment resulted in greater reductions of both blood pressure and markers of oxidative stress. Collectively, these data suggest that both reduce changes driven, in part, by Ang II-mediated increases in NADPH oxidase and, in part, increases in blood pressure.

cardiac remodeling; reduced nicotinamide adenine dinucleotide phosphate oxidase; oxidative stress

INCREASED TISSUE RENIN-ANGIOTENSIN SYSTEM (RAS) activity likely plays a key role in the development of cardiac hypertrophy, remodeling, and hypertension. Components of the RAS, including renin, constitute an autocrine/paracrine system in the myocardium. Angiotensin (Ang) II receptors have been characterized in cardiomyocytes and cardiac fibroblasts (8, 34) as well as the endothelial lining of coronary arteries (46). Conversion of angiotensinogen to Ang I by renin is the rate-limiting step in the generation of Ang II (32). Thus, inhibiting renin activation should abrogate any direct effects of renin as well as reduce tissue Ang II levels/actions. Blockade of the Ang II type I receptor (AT1R) has cardiac protective properties, but AT1R blockade also results in increased release of renin from renal juxtaglomerular cells, which may create a feed-forward mechanism for myocardial injury through cellular actions of renin that do not depend on conversion of angiotensinogen to angiotensin peptides (25). Thus, the use of renin inhibitors, such as aliskiren, hold promise in complementing current inhibitors of RAS activity in the prevention of tissue injury and remodeling by blocking this pathway at its source (27).

Many of the cardiac pathological effects of RAS activation appear to be mediated through redox cycling of reactive oxygen species (ROS), generated primarily by a NADPH oxidase-dependent pathway (4–6). In cardiomyocytes this oxidase is comprised of a membrane-bound NOX2 and p22phox heterodimer and four regulatory subunits, p40phox, p47phox, p67phox, and the small G protein Rac1 (4–6, 9). Activation of this complex promotes hypertrophy and perivascular fibrosis (16, 44). The importance of RAS-induced NADPH oxidase activation and the generation of ROS in this cardiac pathology is supported by the fact that inhibitors of NADPH oxidase (24, 31), free radical scavengers (12, 44), and AT1R blockade (30, 44) attenuate this pathological process.

The new nonpeptide renin inhibitor, aliskiren, is a potent human and mouse renin inhibitor; the IC50 (i.e., the half-maximal inhibitory concentration) for human renin is 0.6 nmol, for mouse renin is 4.5 nmol, and for rat renin is 80 nmol (13, 19). Because of this species specificity for its substrate, aliskiren cannot be studied efficiently in conventional rat models. To circumvent this issue, we have employed the transgenic (mRen2)27 rat (Ren2), which overexpresses the mouse renin transgene in a number of tissues, including the heart (21, 47). This allows for the investigation of renin inhibition in a model of chronic (in utero onward) RAS activation with subsequent elevated tissue levels of Ang II in the heart and other tissues. We have previously utilized this model to evaluate the role of elevated cardiac RAS on NADPH oxidase generation of ROS and resulting structural/functional changes as evaluated by transmission electron microscopy (TEM), histological, immunohistochemical, and biochemical techniques (16, 38, 44). In the current investigation, we hypothesized that renin inhibition employing aliskiren administration in vivo would attenuate the excessive oxidative stress perivascular and interstitial fibrosis...
and mitochondrial abnormalities that have been observed in myocardial tissue of Ren2 rats (11, 16, 28).

MATERIALS AND METHODS

Animals and treatments. All animal procedures were approved by the University of Missouri animal care and use committees and housed in accordance with National Institutes of Health (NIH) guidelines. Male Ren2 (6–7 wk of age) and age-matched male SD rats were randomly assigned to placebo (Ren2-C and SDC), aliskiren (Ren2-A and SDA) at 50 mg·kg⁻¹·day⁻¹ (13), or irbesartan (Ren2-I) at 30 mg·kg⁻¹·day⁻¹ in saline via intraperitoneal injection. Since there have been no reports of comparisons of in vivo BP effects of irbesartan and aliskiren, we estimated, on the basis of available pharmacological data, the dose of irbesartan that would provide a BP-lowering effect comparable with that achieved with aliskiren at the dose used in this study.

Systolic BP. After several simulated systolic BP (SBP) measurements for acclimation of animals, SBP was measured in triplicate, on separate occasions throughout the day, using the tail-cuff method (Student Oscillometric Recorder, Model MC4000 RSP; Harvard Systems) prior to initiation of treatment and on days 19 or 20 prior to the rats being killed (16, 38).

Measurement of NADPH oxidase activity. NADPH oxidase activity was determined in plasma membrane fractions, as described previously (44). Aliquots of membrane and cytosolic fractions (12.5–100 μg proteins) were incubated with NADPH (100 μM) at 37°C. NADPH activity was determined by measuring the conversion of Radial Detector (Cayman Chemical) in the absence and presence of NADPH inhibitor diphenylene iodonium sulfate (50 μM) using spectrophotometric (450 nm) techniques.

Immunostaining of NADPH oxidase subunits. Harvested left ventricle (LV) tissue was immersed and fixed in 3% paraformaldehyde (44). After fixation, tissues were placed in histological cassettes and dehydrated with ethanol series, infiltrated with low-melting (50°C) paraffin, and embedded in high-melting (56°C) paraffin. Blocks were sectioned by 4 μm, deparaffinized in CitriSolv, and rehydrated, and embedded in high-melting (56°C) paraffin. Blocks were sectioned by 4 μm, deparaffinized in CitriSolv, and rehydrated in ethanol series and HEPES wash buffer, and epitopes were retrieved in citrate buffer. The first section was washed (3 × 15 min) with HEPES wash buffer and then mounted with Mowiol (1st control level). The second and third sections were washed and incubated with 1:100 primary and secondary antibodies in 10-fold diluted blocking system overnight. The goat antibody-based sections were incubated with 1:300 goat-anti mouse Alexa fluor 647 in 10-fold diluted blocker. The mouse antibody-based sections were incubated with 1:300 of Alexa fluor goat anti-mouse 647 in 10-fold diluted blocker overnight. After being washed with HEPES wash buffer, the sections were incubated with 1:300 goat-anti mouse Alexa fluor 647. Four hours later the slides were washed and incubated with 1:2,000 4,6-diamidino-2-phenylindole to counterstain nuclei. After 10 min, the slides were washed and mounted with Mowiol. Slides were checked under a laser confocal microscope (Bio-Rad) and a multiphoton confocal system (44). Images were captured with Laser-sharp software and the mitochondria quantified using MetaMorph (Boyce Scientific, St. Louis, MO).

TEM. Heart tissue was thinly sliced and placed in primary EM fixative as described (16). Following secondary fixation, specimens were placed on a rocker overnight, embedded, and polymerized at 60°C for 24 h. Eighty-five-nanometer sections were then stained with 5% uranyl acetate and Satos triple lead stain for viewing by a JOEL transmission electron microscope.

Statistical analysis. All values are expressed as means ± SE. Statistical analyses were performed in SPSS 15.0 (SPSS, Chicago, IL) using unpaired Student’s t-tests or ANOVA with Fisher’s least significant difference test as appropriate.

RESULTS

SBP. At the end of the treatment, Ren2 controls (Ren2-C) had a significantly higher systolic blood pressures compared with SD controls (SD-C; 189.6 ± 3.0 vs. 141.7 ± 0.1, P < 0.05), whereas rats treated with aliskiren (Ren2-A) did not develop further elevations in SBP over baseline levels during the 3 wk of treatment (153.6 ± 4.3 mmHg, P < 0.05 compared with Ren2-C) (Fig. 1). Treatment with irbesartan (Ren2-I) reduced BP more than what was observed with aliskiren treatment (132.1 ± 1.2 mmHg, ∆−21.2 mmHg, P < 0.05).

NADPH oxidase. NADPH oxidase activity was elevated in Ren2 (1.24 ± 0.05 mOD·mg⁻¹·min⁻¹) normalized to SD controls (P < 0.05) (Fig. 2A). This activity was reduced with aliskiren treatment (0.95 ± 0.04 mOD·mg⁻¹·min⁻¹, P < 0.05) and to a greater extent with irbesartan (0.66 ± 0.05 mOD·mg⁻¹·min⁻¹, P < 0.05 compared with Ren2-C and
Ren2-A). NADPH oxidase subunits p47phox and Rac1 were increased in the Ren2 control (75.3 ± 3.8 and 68.6 ± 7.9 average grayscale intensities, respectively) compared with SD control (60.0 ± 5.1 and 54.0 ± 2.7 average grayscale intensities, respectively, P < 0.05) (Fig. 2, B and C). There were reductions in both subunits in the aliskiren-treated Ren2 (55.8 ± 0.1 and 50.6 ± 1.6 average grayscale intensities, respectively, P < 0.05), and these reductions were even greater with irbesartan treatment (39.8 ± 2.9 and 37.3 ± 3.3 average grayscale intensities, P < 0.05 compared with Ren2-C and Ren2-A).

Oxidative stress measures. Immunostaining for 3-nitrotyrosine, a surrogate marker of oxidative and nitroso stress (13), was elevated in Ren2-C normalized to SD-C (1.53 ± 0.12 average grayscale intensities, P < 0.05; Fig. 3). 3-Nitrotyrosine levels were reduced with aliskiren treatment (1.14 ± 0.07 average grayscale intensities, P < 0.05), and this reduction was even greater with irbesartan (0.76 ± 0.13 average grayscale intensities, P < 0.05 compared with Ren2-C and Ren2-A).

Myocardial remodeling. VVG staining was used to evaluate perivascular fibrosis in LV tissue. Perivascular fibrosis, represented by percent adventitial area per vessel, was significantly increased in the untreated Ren2-C compared with the SD-C (61.1 ± 2.4 vs. 44.7 ± 2.6%, P < 0.05) and abrogated by both aliskiren (45.4 ± 2.4%, P < 0.05) and irbesartan treatment (45.5 ± 2.4%, P < 0.05) (Fig 4A).

Ultrastructural analysis of the myocardium utilizing TEM revealed qualitatively abnormal intercalated discs (ID) in the Ren2 left ventricle. There was a prominent appearance of ID duplication in the Ren2-C compared with the SD-C (Fig 4C). Duplication of the ID may reflect an increasing number of convolutions to compensate for the lengthening of this structure and the increase strength of adhesion with adjacent myofibrils (26). ID duplication

Fig. 1. Systolic Blood Pressure (SBP) in the transgenic Ren2 rat. SBP was measured prior to the experimental protocol being started and on days 19 and 20 prior to the rats being killed (day 21). Values are presented as means ± SE. *P < 0.05 compared with Sprague-Dawley control (SD-C); **P < 0.05 compared with Ren2 control (Ren2-C); #P < 0.05 compared with aliskiren-treated Ren2 (Ren2-A). SD-A, aliskiren-treated Sprague-Dawley; Ren2-I, Ren2 rats treated with irbesartan.

Fig. 2. Myocardial NADPH oxidase in the transgenic Ren2 rat NADPH oxidase activity (A) and immunostaining of subunits p47phox and Rac1 and quantification of converted signal intensities in average grayscale intensities to the bottom (B and C). *P < 0.05 compared with SD-C; **P < 0.05 compared with Ren2-C; #P < 0.05 compared with aliskiren-treated Ren2 (Ren2-A). Scale bar, 50 um.
was qualitatively diminished by treatment with both renin inhibition and AT1R blockade. Both treatments normalized the ultrastructural appearance of the myocardial tissue, resulting in fewer convolutions and less ID duplication.

Mitochondria. Quantification via immunostaining for mitochondria-specific protein complex IV subunit also revealed significant increases in the Ren2-C normalized to SD-C (2.47 ± 0.51 relative number, $P < 0.05$; Fig. 5, A and B) as
well as attenuation of this phenomena following renin inhibition ($1.15 \pm 0.13$ relative number, $P < 0.05$) and irbesartan treatment ($1.14 \pm 0.13$ relative number, $P < 0.05$ compared with Ren2-C). Mitochondrial biogenesis was evaluated by TEM (16, 43) and quantified with immunostaining. 10K TEM images (Fig. 5C) of the Ren2-C heart revealed striking changes in the mitochondria. There were increased numbers of smaller mitochondria as well as increased convolutions (duplications) of ID in Ren2-C compared with the SD-C myocardial tissue (Fig. 5A). The structurally abnormal convoluted ID of the Ren2-C also seem to interfere with the normal orderly linear arrangement of the myofibrils. There was also an increase in complex IV (cytochrome c oxidase) in the mitochondria of Ren2 myocardial tissue reflecting overall increases in mitochondrial activity, likely a compensatory response to establish a transmembrane differential proton electrochemical potential used to generate ATP. Thus these mitochondrial changes likely reflect increased energy requirements in the untreated Ren2 myocardium (20). Following treatment with both the renin inhibitor and the AT1R blocker, the mitochondria number structural makeup and complex IV levels were similar to those observed in SD control myocardium.

**DISCUSSION**

The purpose of this investigation was to evaluate the impact of two treatments to reduce tissue RAS, renin inhibition and AT1R blockade, on cardiac oxidative stress and remodeling in a transgenic rat manifesting mouse renin transgene overexpression. Compared with its SD littermate, Ren2 rats are characterized by increased plasma and tissue total renin, prorenin, and Ang II despite lower kidney levels of renin (7). Previous reports have implicated elevated tissue Ang II levels in the activation of NADPH oxidase, which produces increased levels of oxidative stress that contribute to cardiac remodeling and dysfunction (4, 6, 16). Similarly to previous work with AT1R blockade (44), results of this investigation show that in vivo inhibition of renin, the rate-limiting step in Ang II formation, abrogates RAS-mediated enhanced activation of NADPH oxidase/increased oxidative stress and reduces left ventricular perivascular fibrosis, mitochondrial content, and remodeling. Although aliskerin and irbesatan had similar overall effects, irbesartan reduced SBP and parameters of oxidative stress to a greater extent than aliskerin in this investigation. Indeed, there is evidence that the degree of BP lowering, independently of RAS effects, irbesartan reduced SBP and parameters of oxidative stress to a greater extent than aliskerin in this investigation. Indeed, there is evidence that the degree of BP lowering, independently of RAS effects, may impact reductions in cardiovascular NADPH oxidase activity (42). Thus, it is likely that the greater BP-lowering effects of irbesartan, in the dose used in this study, accounted for the greater lowering of NADPH oxidase activity.

The heart has a limited endogenous antioxidant capacity, which renders it highly susceptible to ROS-induced tissue damage (12). In fact, oxidative stress has been causally associated with cardiac hypertrophy, remodeling, and ventricular dysfunction (10, 38, 44). Data from our laboratory have demonstrated that myocardial activation of NADPH oxidase and resultant increases in oxidative stress contribute to remodeling and dysfunction (16, 44). This is critical to attribute, in large part, due to the structural and functional changes observed with...
oxidative stress rather than physical stress induced by hyper-tension.

Immunostaining for 3-nitrotyrosine demonstrated that left ventricular tissues harvested from untreated heterozygous male Ren2 transgenic rats manifested significantly increased NADPH oxidase activity and ROS compared with age-matched SD controls. Three weeks of treatment with aliskiren and irbesartan effectively attenuated ROS and reactive nitrogen species generation that has been directly implicated in Ang II-induced cardiac hypertrophy and fibrosis (24, 31, 37). In accordance with previous studies (16, 38, 43), histological examination using VVG staining showed prominent increases in myocardial perivascular fibrosis and ultrastructural remodeling in the untreated Ren2. Both structural and biochemical changes were normalized by 3 wk of both aliskiren and irbesartan treatment. Thus, both renin inhibition and AT1R blockade in young Ren2 rats effectively attenuated myocardial NADPH oxidase-overactivity and reduced markers of oxidative stress; this was accompanied by reduced myocardial fibrosis and cellular ultrastructural TEM abnormalities.

TEM showed that heterozygous male Ren2 hearts possess increased numbers of smaller mitochondria, consistent with staining for mitochondrial protein, as published previously from our laboratory (38, 44). There were also increased convolutions (duplication) of myocardial ID, suggesting a structural response to increased mechanical requirements in Ren2 heart tissue. Increased numbers of smaller mitochondrial are suggestive of altered mitochondrial function and biogenesis/or enhanced energy requirements in hearts subjected to excessive oxidative stress and cardiac afterload (17, 26, 36). Additionally, the increased convolutions (duplication) of the ID observed may also serve to increase contact between cardiomycytes as an adaptive response to increased cardiac energy requirements and reduced contractile efficiency accompanying increased afterload (38, 44). The reduction in complex IV with renin inhibition and AT1R blockade may reflect a lesser work load on the Ren2 heart following either RAS inhibition therapeu-tic strategy.

Oxidative stress can adversely affect mitochondria through numerous mechanisms, such as mitochondrial DNA (mtDNA) damage (26, 36). Damaged mtDNA, such as mutant mtDNA with large deletions, has been shown to quickly and predominantly repopulate cellular mitochondria (17). Additionally, low myocardial ATP concentrations, due to dysfunctional mitochondria, have been shown to stimulate proliferation of defective mitochondria in an attempt to meet the energy require-ments of the tissue (17, 26, 36). This notion is consistent with the idea that mitochondria in highly oxidative environments generally proliferate more rapidly and thus tend to be smaller and more plentiful. This concept is reinforced by our observations that these mitochondrial morphological, as well as functional, alterations are markedly attenuated, in conjunction with contemporaneous reductions in oxidative stress, in young Ren2 rats treated following in vivo treatment with agents reducing RAS activity.

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REFERENCES


